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Analytical Methods

Development of a novel biosensor based on F_0F_1-ATPase for the detection of 2-dodecylcyclobutanone in irradiated beef

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ABSTRACT

A novel biosensor regulated by the rotator of F_0F_1-ATPase was developed to analyze 2-dodecylcyclobutanone (2-DCB) to detect γ-ray irradiated beef rapidly. The biosensor was assembled by conjugating 2-DCB monoclonal antibodies with the "rotator" ε-subunit of F_0F_1-ATPase within chromatophores through an ε-subunit monoclonal antibody–biotin–avidin–biotin linker. The limit of detection (LOD) of 2-DCB was approximately 10^{-8} μg/mL. The recovery ratio of 2-DCB from ground beef patties ranged from 75.1% to 116.4%. The intra-assay and inter-assay coefficients of variation were both <15.0%. The proposed method was validated by gas chromatography–mass spectrometry with high correlation. The biosensor was used to detect 2-DCB in ground beef patties with different fat contents (10%, 20%, and 30%) irradiated at 0.5, 1.0, 3.0, 5.0, and 7.0 kGy. The 2-DCB concentration linearly increased with the radiation dose in all the beef samples. 2-DCB concentration increased with fat levels in the three samples.

1. Introduction

Irradiation of food improves food safety and maintains food quality by controlling microorganisms and extending shelf life (Gadgil, Smith, Hachmeister, & Kropf, 2005). Although this process is considered a safe food preserving method, to comply with both national and international legislation and to enhance consumer confidence, it is necessary to develop a reliable, convenient, and rapid method to distinguish irradiated from non-irradiated products (Hijaz, Kumar, & Smith, 2010). 2-Alkylcyclobutanones are radiolytic products that include 2-dodecylcyclobutanone (2-DCB) from palmitic acid and 2-tetradecylcyclobutanone (2-TCB) from stearic acid; these compounds have been used to detect irradiated lipid-containing foods (Blanch, Caja, Flores, & Castillo, 2009; Elliott, Hamilton, Stevenson, McCaughey, & Boyd, 1995; Gadgil, Hachmeister, Smith, & Kropf, 2002; Horvatovich, Miesch, Hasselmann, Delinceée, & Marchioni, 2005; Obana, Furuta, & Tanaka, 2005; Soncin et al., 2012; Zhao, Wang, Li, Li, & Ha, 2012) 2-Alkylcyclobutanones are considered unique radiolytic products because they are not formed during cooking or any other heat-processing methods.

The analytical method EN 1785 based on mass spectrometric detection of 2-DCB and 2-TCB after gas chromatographic separation (GC–MS), adopted by the European Committee for Standardization as an official method (European Standard, EN1785) (Anonymous, 2003), has been widely used for the identification of irradiated food. The method comprises three steps: fat extraction using Soxhlet extraction method, followed by adsorption chromatography cleanup, and then chromatographic analysis via GC/MS, which is accurate but expensive and time-consuming (Elliott et al., 1995). Recently, ^{1}H NMR lipid profiling and metabolite profiling has been applied to distinguish irradiated beef from non-irradiated ones (Zanardi et al., 2013, 2015). The method is promising because of the simple sample preparation procedure and decreased analysis time. However, beef samples irradiated at doses lower than 2.5 kGy cannot be identified using the method currently and it needs a relative large number of samples to validate the technique and to classify new samples. Therefore, a novel simple and fast method is required for rapid 2-DCB detection to identify irradiated lipid-containing foods.

F_0F_1-ATPase, a membrane-bound holoenzyme in chloroplasts, bacteria, and in the mitochondria, is a ubiquitous rotary motor that have two components, F_0 ($a_1b_2c_n$) and F_1 ($\alpha_3\beta_3\gamma_1\delta_1\varepsilon_1$), which are mechanically coupled by a common central stalk ("rotor"), $c_n\varepsilon\gamma$ (Yagi et al., 2007). The membrane-embedded F_0 unit converts the electrochemical potential of protons (or sodium ions in some bacteria) across membrane into mechanical rotation of the "rotor"

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$c_n\varepsilon\gamma$, forcing a cyclic conformational change in the $\alpha_3\beta_3$ crown (“stator”) of F_1, resulting in ATP synthesis from adenosine diphosphate (ADP) and inorganic phosphate (Pi) (Imamuraa et al., 2009; Liu, Zhang, Yue, Jiang, & Zhang, 2006; Noji & Yoshida, 2001; Okuno, Iino, & Noji, 2011). Previous investigations (Zhao et al., 2012) have demonstrated that the holoenzyme activity can be regulated by external links on the “rotator” ε-subunit. ATP synthetic activity was activated with external complexes bind to the “rotor” ε-subunit. Therefore, F_0F_1-ATPase has great potential for designing rapid, free-labeled, sensitive, and selective immuno-rotary biosensors.

In this paper, a biosensor regulated by the “rotator” ε-subunit of F_0F_1-ATPase was developed to analyze 2-DCB to detect γ-ray irradiated beef for the first time. The biosensor developed in this paper is a potential alternative to chromatography or spectrometry for the regulatory analysis of 2-DCB in irradiated lipid-containing foods.

2. Materials and methods

2.1. Chemicals and reagents

Standard 2-DCB and 2-TCB, ADP, and neutroavidin were purchased from Sigma–Aldrich (Buchs, Switzerland). Hexane was purchased from Fisher Scientific (Pittsburg, PA, USA). Acetonitrile, 2-ethylcyclobutanone, 2-oxo-cyclobutane, carboxylic acid, tridecanoic acid, *n*-tridecane, and anhydrous sodium sulfate were obtained domestically. Mega Bond Elut SI, 1 g/6 mL were obtained from Varian Inc. (Palo Alto, CA, USA). ENLITEN Luciferase/Luciferin Reagent was purchased from Promega, USA. The microplate luminometer was a Centro XS3 LB 960 (Germany). Chromatophores containing F_0F_1-ATPase, biotinylated ε-subunit monoclonal antibodies, ATP synthesis buffer, and biotinylated 2-DCB monoclonal antibodies were prepared according to previous study (Zhao, Wang, Li, & Ha, 2013; Zhao et al., 2012). All other analytically purified reagents were purchased domestically.

2.2. Construction of immuno-rotary biosensor (IRB)

The IRB was constructed according to previous study (Zhao et al., 2012) with slight modifications. Approximately 15 μL of chromatophores (50 mg/mL) and 8 μL (0.5 mg/mL) of biotinylated ε-subunit monoclonal antibodies were mixed in 1 mL of PBS buffer, and incubated at 37 °C for 60 min. Then, the free ε-subunit monoclonal antibodies were washed away by centrifugation at 40,000×*g* for 20 min at 4 °C. The precipitate was resuspended in 500 μL of PBS buffer. Then, 7.5 μL (0.1 mg/mL) of neutroavidin was added and diluted into 1 mL with PBS buffer, and incubated at 37 °C for 10 min. The free neutroavidin was washed away by 20 min centrifugation at 40,000×*g* at 4 °C. The precipitate was resuspended in 500 μL of PBS buffer. Then, 28 μL (0.036 mg/mL) of biotinylated 2-DCB monoclonal antibodies were added and diluted into 1 mL with PBS buffer, and incubated at 37 °C for 10 min. The free 2-DCB monoclonal antibodies were then washed away by centrifugation at 40,000×*g* for 20 min at 4 °C. The precipitate was resuspended in 500 μL of PBS buffer. The IRB are stable at −20 °C for at least 1 year. Avoid repeated freeze–thaw cycles.

2.3. ATP synthesis activity assay of F_0F_1-ATPase within the chromatophores

The ATP synthesis activity of F_0F_1-ATPase within the chromatophores was determined using the luciferin–luciferase method via a microplate luminometer according to previous study (Zhao et al., 2012) with slight modifications. Up to 10 μL of the F_0F_1-ATPase molecular motor biosensors and 30 μL of ATP synthesis buffer were mixed together and incubated at 37 °C for 10 min. After the reaction was terminated by adding 450 μL of PBS, 50 μL of dilution buffer was added to 30 μL of the luciferase/luciferin reagent, and was mixed. The chemiluminescence was then immediately detected using a microplate luminometer.

2.4. Sample pretreatment for biosensor test

The sample was pretreated according to previous study (Zhao et al., 2013) with slight modifications.

Fresh chilled ground beef patties (10%, 20%, and 30% fat) packed in polyethylene packs were purchased from local markets in Beijing and were irradiated at 0.5, 1.0, 3.0, 5.0 and 7.0 kGy using a ^{60}Co radiation source at room temperature in the radiation center of the Chemistry Institute of Peking University, and then were immediately stored at −20 °C. A 5 g sample of each set of ground beef patties was homogenized with 10 g of anhydrous sodium sulfate. The homogenate was blended with 100 mL acetonitrile using a tissue-mashing instrument for 1 min. The mixture was left to stand for 1 min before it was blended again for another 1 min. The extraction solvent was carefully passed through filter paper as it was transferred to a 500 mL round-bottomed flask. The reaction mixture was retained in the tissue-mashing instrument. Another 100 mL acetonitrile was then added, and the entire extraction procedure was repeated, as described above. Finally, the extract solutions were combined and evaporated using a rotary evaporator until dry. The round-bottomed flask was washed with hexane, and the obtained solution was collected into a 50 mL volumetric flask. A 10 mL Hexane (10 mL) was evaporated to 1 mL via a nitrogen stream, added to a silica SPE cartridge column, and immediately rinsed with 10 mL of *n*-hexane for conditioning before use. A 10 mL *n*-hexane aliquot was eluted and discarded. Subsequently, a 10 mL aliquot of a 2% diethyl ether-*n*-hexane solution (2:98, v/v) was eluted and collected from the 2-alkylcyclobutanone fraction. The eluted solution was concentrated using a nitrogen stream until dry, and resuspended in 50 μL methanol followed by 450 μL of a PBS buffer (pH 7.4). A 10 μL aliquot of the resulting solution was then assayed by the biosensor. For the GC–MS analysis, after concentrated to dryness, the remaining eluate was resuspended in 500 μL of *n*-hexane. A 1 μL aliquot of the resulting solution was subsequently used in the GC–MS analysis.

2.5. Detection of 2-DCB solution using the biosensor

The presence of 2-DCB was detected using the novel biosensor, as indicated by the change in fluorescence intensity through ATP synthesis by the F_0F_1-ATPase within the chromatophores. 10 μL of the F_0F_1-ATPase molecular motor biosensors labeled with 2-DCB monoclonal antibodies were mixed with 10 μL of 2-DCB solution/pretreated sample solution. After 10 min, the ATP synthetic activity of 10 μL of the mixture was determined.

2.6. Validation of the biosensor method by GC–MS analysis

To validate the proposed biosensor, the 2-DCB spiked ground beef patties were analyzed using a QP2010 Plus model GC–MS system (Shimadzu, Kyoto, Japan). The GC conditions were according to previous study (Zhao et al., 2012) as follows: column, Rtx-5MS (30.0 m × 0.25 μm × 0.25 mm); column temperature program, 55 °C (1 min), 55 °C to 180 °C at 10 °C/min, 180 °C to 250 °C at 2 °C/min, 250 °C (2 min); carrier gas, helium at 1.00 mL/min; injection temperature, 250 °C; injection mode, splitless; and injection volume, 1 μL. The MS conditions were as follows: ionization mode, electron ionization; ion detection, selected ion monitoring (SIM);

detector voltage, 0.8 kV; ion source temperature, 200 °C; and transfer line temperature, 250 °C. The monitored ions were *m*/*z* 98 and 112; *m*/*z* 98 was likewise selected for determination.

3. Results and discussion

3.1. Optimization of the rotary biosensor conjugation conditions

Fig. 1 (adapt from Zhao et al., 2012 with slight modifications) shows the basic design of the novel rotary biosensor based on F_0F_1-ATPase within a chromatophore. 8 μl (0.5 mg/mL) of the ε-subunit monoclonal antibodies and 7.5 μl (0.1 mg/mL) of neutroavidin were linked to F_0F_1-ATPase as described previously (Zhao et al., 2012). Then, the ATP synthetic activity of the F_0F_1-ATPase regulated by binding different quantities of 2-DCB monoclonal antibodies was studied in detail to optimize the construction conditions of the biosensor. Fig. 2 shows the average fold-increase in relative fluorescence intensity caused by the loading of biotinylated 2-DCB monoclonal antibodies onto the F_0F_1-ATPase within the chromatophores. The biotinylated ε-subunit monoclonal antibodies were linked with F_0F_1-ATPase as described in the Materials and methods section, followed by neutroavidin, and the mixture obtained was used as control. Then the biotinylated 2-DCB monoclonal antibodies were linked. The fluorescence intensities, which reveal a good correlation with the ATP synthetic activity, stabilized when the amount of biotinylated 2-DCB monoclonal antibodies was 28 μl (0.036 mg/mL), which was caused by the quantitative limitation in chromatophores. This result indicates that the 2-DCB monoclonal antibodies were successfully linked with the ε-subunit of the F_0F_1-ATPase within the chromatophores and 28 μl (0.036 mg/mL) of 2-DCB monoclonal antibodies was optimum for conjugation.

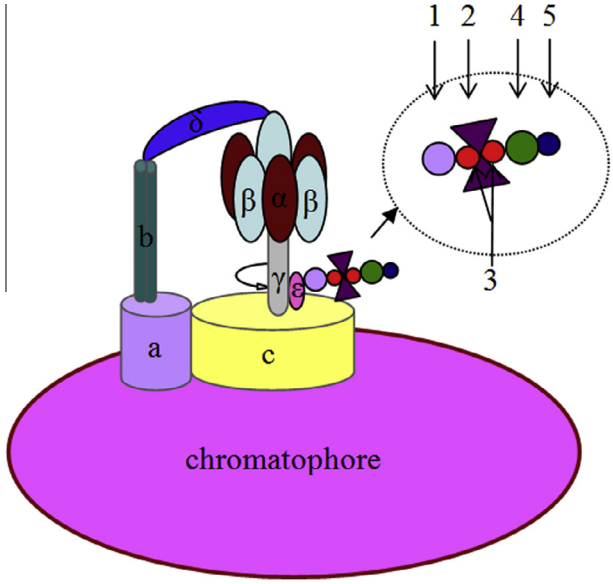

Fig. 1. The schematic illustration of immuno-rotary biosensor based on F_0F_1-ATPase. (1) ε-Subunit monoclonal antibody; (2) neutroavidin; (3) biotin; (4) 2-DCB monoclonal antibody; (5) 2-DCB molecular (adapt from Zhao et al., 2012 with slight modifications).

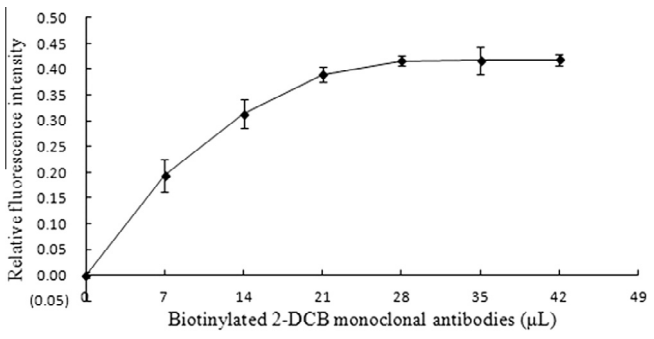

Fig. 2. Intensity change of relative ATP synthesis activities of the F_0F_1-ATPase caused by the loading of biotinylated 2-DCB monoclonal antibody onto F_0F_1-ATPase within the chromatophores (n = 3).

3.2. Characterization of the assay

Under optimal assay conditions, the sensitivity and specificity of the proposed biosensor were investigated. The standard curve of the biosensor for 2-DCB determination in buffer solution was constructed at concentrations from 0.0001 μg/mL to 100 μg/mL in the buffer solution (Fig. 3). This curve could be described using the linear equation $y = 0.0379x + 0.3353$, $R^2 = 0.953$. The limit of detection (LOD), calculated as ten times the mean value of the blank buffer solution, was approximately 10^{-8} μg/m, thereby indicating high sensitivity of the assay. In addition, 2-DCB can be determined for only about 10 min using the proposed biosensor without blocking, labeling, and washing procedure.

The specificity of the nanoscale biosensor was assessed by detecting five structurally related compounds (2-TCB, 2-ethylcyclobutanone, 2-oxocyclobutanecarboxylic acid, tridecanoic acid, and *n*-tridecane) simultaneously. The results show that the relative fluorescence intensity caused by loading 10 μL of blank buffer solution which was used as the control was 0.00193 ± 0.00027. The relative fluorescence intensity caused by loading 10 μL of the five structurally related compounds at 1 μg/mL were 0.00279 ± 0.00045, 0.00196 ± 0.00042, 0.00182 ± 0.00063, 0.00165 ± 0.00054, and 0.00192 ± 0.00038, respectively. There was no significant difference compared with the control, which indicates that the five structurally related compounds do not have any direct effect on the biosensor.

3.3. Accuracy and precision of the assay

To test the accuracy and precision of the proposed biosensor, ground beef patties were spiked with 2-DCB at concentrations of 0.01, 0.1, and 0.5 mg/kg of sample to determine recovery, intra-assay variation, and inter-assay variation of each treatment. Unspiked samples were used as blanks. The samples were extracted with acetonitrile via direct solvent extraction with a tissue-mashing instrument before they were purified using a 1 g silica cartridge. The elution was concentrated to dryness,

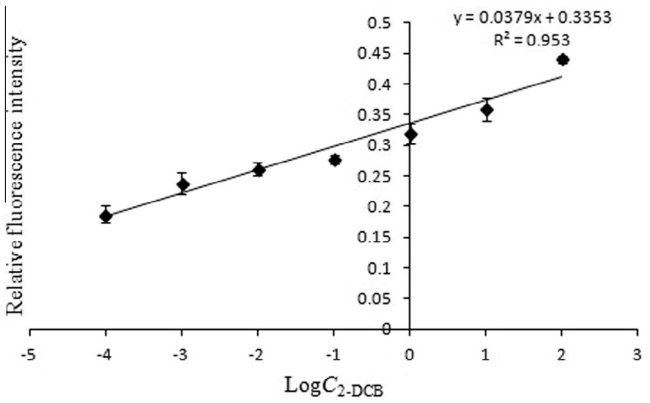

Fig. 3. The standard curve of 2-DCB (n = 3).

resuspended in 50 μL methanol, and diluted with 450 μL PBS (pH 7.4) to completely eliminate the matrix effect. The diluted extracts were then analyzed by the biosensor. 2-DCB was not detected in unspiked samples. The recovery ratios were calculated using the standard calibration curve; the results are shown in Table 1. The biosensor gave acceptable recovery ratios in the range of 75.1% to 116.4%. The intra-assay and inter-assay coefficients of variation were both <15.0, thereby demonstrating the high accuracy and precision of the assay.

Table 1
Results of the recovery, inter-assay variation, and intra-assay variation of ground beef patties spiked with 2-DCB ($n = 3$) using the biosensor.

Concentration fortified (mg/kg of beef)	Average recovery (%)	Intra-assay variation[a] (%)	Inter-assay variation[b] (%)
0.01	106.5	11.2	12.1
0.1	75.1	12.4	14.4
0.5	116.4	5.8	13.8

[a] Intra-assay variation was determined by analyzing one extraction sample on a single day three times.
[b] Inter-assay variation was determined by analyzing one extraction sample with three independently constructed IRB.

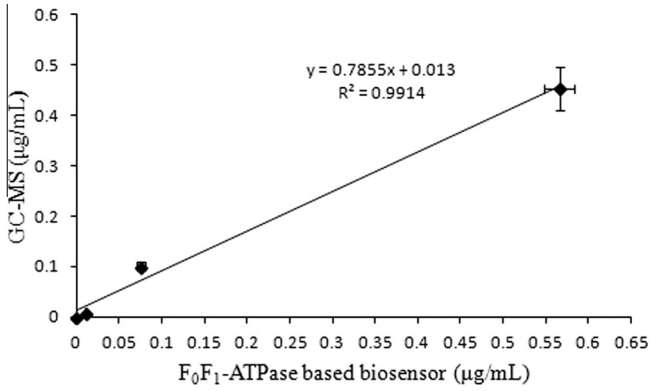

Fig. 4. Correlation between the proposed biosensor and GC–MS for ground beef patties spiked with 2-DCB at concentrations of 0.01, 0.1 and 0.5 mg/kg beef ($n = 3$).

3.4. Validation of the biosensor method by GC–MS

To evaluate the quality of the biosensor developed in our research, ground beef patties spiked with 2-DCB at concentrations of 0.01, 0.1 and 0.5 mg/kg were simultaneously analyzed by GC–MS and biosensor. 2-DCB was independently extracted from the beef samples and cleaned up before 2-DCB measurements by GC–MS and biosensor. The GC–MS calibration curve for 2-DCB was constructed in 0.01, 0.02, 0.05, 0.10, 0.50, and 1.0 μg/mL; its linear equation was $y = 263175.6x - 2513.994$ ($R^2 = 0.9995$). As shown in Fig. 4, the results from these two methods were highly correlated. The linear regression equation of $y = 0.7855x + 0.013$, $R^2 = 0.9914$ indicated the reliability of the proposed biosensor test. These results suggested that 2-DCB in food samples could be simply, rapidly, and accurately detected by the biosensor.

3.5. 2-DCB content of irradiated ground beef patties

The proposed biosensor was used to evaluate the amount of 2-DCB in the ground beef patties with different fat contents (10%, 20% and 30%) irradiated at 0.5, 1.0, 3.0, 5.0, and 7.0 kGy. 2-DCB was not detected in the non-irradiated beef samples. The linear relationship between the irradiation dose and the 2-DCB content is presented in Fig. 5. 2-DCB concentration increased with fat levels in the three samples. This result agrees with similar studies on the effect of the irradiation dose on the 2-DCB concentration (D'oca et al., 2009; Tewfik, 2008). However, an exponential increase was observed between 2-ACBs content and irradiation dose in the study of irradiating of three cured meat products salame Milano, pancetta, and coppa (Zanardi et al., 2007). The inconsistency between the relationship observed in this study and our study could be ascribed to the ingredients and additives with pro-oxidant (sodium chloride) and antioxidant (sodium nitrate, sodium nitrite, and ascorbic acid) properties that are present in cured meat products, but absent in raw meat. Because the additives may affect the formation of 2-ACBs in cured pork products by scavenging the radicals formed during irradiation depending on the irradiation dose.

4. Conclusions

A F_0F_1-ATPase based biosensor was developed to analyze 2-DCB to detect γ-ray irradiated beef. The limit of detection (LOD) of the optimized biosensor was 10^{-8} μg/mL. Interferences caused by

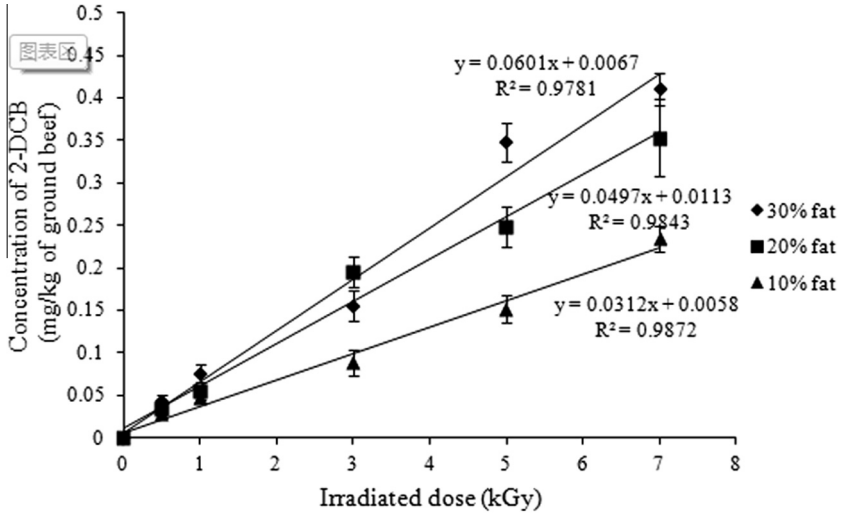

Fig. 5. Effect of irradiation dose and fat level on the concentration of 2-DCB in irradiated ground beef patties ($n = 3$).

sample matrix were easily overcome by a simple dilution step before analysis. After sample pretreatment, 2-DCB in the irradiated ground beef patties can be determined for only about 10 min using the proposed biosensor without blocking, labeling, and washing. The recovery ratio was 75.1% to 116.4%, whereas the intra-assay and inter-assay coefficients of variation were both <13.0, thereby demonstrating the high accuracy and precision of the assay. The proposed method was validated by its good correlation with GC–MS results. The proposed method was likewise used to detect 2-DCB in ground beef patties irradiated at 0.5, 1.0, 3.0, 5.0, and 7.0 kGy. The concentration of 2-DCB linearly increased with the irradiation dose. In addition, 2-DCB concentration increased with fat levels in the three samples. The proposed biosensor has high sensitivity, high simplicity, rapid reaction time, low cost, and high sample throughput. Thus, the biosensor could be used as a feasible quantitative screening method for the 2-DCB analysis of irradiated ground beef and other lipid-containing foods.

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